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HYDROLYSIS OF GLYCOL CHITIN BY CHITINOLYTIC ENZYMES*

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Abstract—1. The hydrolysis of glycol chitin preparations by several β-*N*-acetylglucosaminidases was monitored colorimetrically with the potassium ferriferrocyanide reagent.

2. Glycol chitin samples from crab and insect sources varied considerably in chemical composition and susceptibility to enzymatic hydrolysis.

3. Insect endochitinase preferred crab glycol chitin as substrate while hen's egg white lysozyme preferred commercial glycol chitin.

4. Insect glycol chitin was well hydrolyzed by both enzymes.

5. Insect exochitinase did not digest glycol chitin.

INTRODUCTION

There are many substrates available for assaying chitinolytic enzymes such as hen's egg white lysozyme and insect endochitinase. They include chitin, ^{3}H-chitin, chitin red®, glycol chitin and $\beta(1\rightarrow4)$ linked oligosaccharides of *N*-acetylglucosamine (Imoto *et al.*, 1972; Kramer *et al.*, 1983). The larger substrates are usually insoluble and frequently contain undefined chemical substituents such as amino acids, peptides or proteins (Brine and Austin, 1981a,b). As a result they may not be very susceptible to enzyme hydrolysis. Glycol chitin, a soluble but chemically modified form of chitin, has been widely used to study lysozyme catalysis (Imoto *et al.*, 1972). We have utilized it to monitor the isolation of endochitinases from the tobacco hornworm, *Manduca sexta* (L.) (Koga *et al.*, 1983). During the course of these studies we have noticed a wide variability in the susceptibility of glycol chitin preparations to chitinolytic enzymes. Here we describe the suitability of crab, insect and commercially prepared glycol chitin samples for use in chitinolytic enzyme assays. The enzymes tested included endochitinase and exochitinase from insect integument and lysozyme from hen's egg white.

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MATERIALS AND METHODS

Materials

M. sexta endochitinase and exochitinase were prepared from molting fluid or integument by the methods of Koga *et al.* (1983) and Dziadik-Turner *et al.* (1981), respectively. Twice crystallized lysozyme was obtained from Worthington Biochemical Co. Commercial glycol chitin was purchased from Sigma (sample A) and Miles (sample B). Crab chitin was from Sigma. Insect chitin (2.7 g) was prepared from lyophilized fifth stadium scraped larval cuticle (20 g) of *M. sexta* by boiling in 1 N NaOH for 15 hr. Glycol chitin was prepared from crab and insect chitin by glycolation with ethylene oxide followed by acylation with acetic anhydride (Senju and Okimasu, 1950; Yamada and Imoto, 1981). A suspension of finely ground chitin was kept for 4 hr at room temperature in 40% NaOH *in vacuo* to promote penetration of alkali into the chitin particles and then filtered. The alkaline chitin was mixed with crushed ice to give a highly viscous solution and diluted with aqueous NaOH to give a 14% NaOH solution. Treatment with ethylene oxide yielded water soluble glycol chitin which was reacetylated with acetic anhydride. Analyses for C, H, O and N were performed by Huffman Laboratories, Wheatridge, Colorado.

Enzyme assay

Enzymes were assayed for glycol chitin hydrolytic activity in 50 mM sodium phosphate pH 6.4. Glycol chitin (0.5 ml of 0.05% solution) was mixed with enzyme (0.05 ml) and incubated at 30°C for 2 hr. The production of reducing end groups was followed colorimetrically with the potassium ferriferrocyanide reagent at 420 nm (Imoto and Yagishita, 1971). Under these conditions the decrease in absorbance caused by incubation with lysozyme and endochitinase was a linear function of time over a period of three hours.

RESULTS AND DISCUSSION

Chitin samples differ in chemical and physical properties depending on the animal source and method of preparation. We have prepared glycol

Table 1. Elemental analysis of chitin, chitosan and glycolated derivatives

Sample	C	H	O	N	N/C
NAcGlc or chitin ($C_8H_{13}O_5N$)	47.29	6.40	39.41	6.89	0.146
Glycol NAcGlc or glycol chitin ($C_{10}H_{17}O_6N$)	48.58	6.88	38.87	5.67	0.117
$GlcNH_2$ or chitosan ($C_6H_{11}O_4N$)	44.72	6.83	39.75	8.70	0.194
Glycol $GlcNH_2$ or glycol chitosan ($C_8H_{15}O_5N$)	46.83	7.32	39.02	6.83	0.146
Crab glycol chitin					
Native	39.17	6.06	41.73	6.58	0.168
Reacetylated	42.48	6.71	38.72	5.69	0.134
Insect glycol chitin					
Reacetylated	42.83	6.79	40.95	5.47	0.128
Commercial glycol chitin					
A	45.96	7.07	39.83	5.16	0.112
B	47.03	6.95	40.03	5.55	0.118

chitins from crab and the tobacco hornworm using the same procedure and compared their elemental compositions with those from commercial glycol chitin samples (Table 1). Also presented in Table 1 is the ratio of N:C from which the degree of glycolation can be estimated (Yamada and Imoto, 1981). This ratio is independent of the degree of hydration of the samples and varies from 0.117 for glycol chitin through 0.146 for chitin or glycol chitosan to 0.194 for chitosan. The samples differed widely in their elemental compositions. This result suggests that noncarbohydrate components may be present in some of the preparations. The values for H and O in Table 1 reflect the presence of trace amounts of moisture in the samples.

Crab glycol chitin yielded a N:C ratio of 0.168 which indicates that glycolation is incomplete or that glucosamine residues are present. Because deacetylation occurs under the alkaline condition of glycolation, the crab glycol chitin was acetylated with acetic anhydride. This treatment reduced the N:C ratio to 0.134 which suggested that acylation occurred yielding a sample with a higher proportion of acetylated glycol chitin present. Reacetylated insect glycol chitin (N:C = 0.128) was apparently very similar to the crab glycol chitin while commercial glycol chitin gave the expected N:C ratio (N:C = 0.112).

We next determined whether there was a correlation between the elemental composition of the glycol chitin samples and their susceptibility to enzymatic hydrolysis (Table 2). We utilized three chitinolytic enzymes including lysozyme, endochitinase and exochitinase. The former two enzymes hydrolyze interior glycosidic bonds while the latter cleaves off monosaccharide units from the nonreducing end of the substrate (Imoto *et al.*, 1972; Koga *et al.*, 1982, 1983). Native and reacetylated crab glycol chitin were the best substrates for insect endochitinase followed by insect glycol chitin and one of the commercial glycol chitins (sample B). The other commercial sample (A) was not hydrolyzed by endochitinase, but it was the most susceptible substrate for hen's egg white lysozyme. Lysozyme digested both commercial samples and insect glycol chitin but it did not hydrolyze crab glycol chitin well using our assay conditions. As expected none of the glycol chitin substrates were attacked to a significant extent by insect exochitinase.

There was no apparent relationship between the chemical compositions of glycol chitin samples and their susceptibility to chitinolytic enzymes. Commercial glycol chitin was expected to be the best substrate for both lysozyme and endochitinase. However the latter preferred crab glycol chitin whose N:C ratio indicated chemical heterogeneity. Although substrates may appear to be similar chemically, they may vary greatly in their degree of heterogeneity and susceptibility to related enzymes. Thus, only by empirical testing can a glycol chitin preparation be found suitable or not as a substrate for the β-*N*-acteylglucosaminidases examined here. The susceptibility of these polymeric substrates may result in part from polymer length, the extent and location of hydroxyethyl functions and other undefined components whose presence is or is not conducive for catalysis. The oligosaccharide chitin substrates may be more reliable for chitinolytic activity determinations because they are prepared under more controlled conditions and are more well defined chemically (Koga *et al.*, 1982, 1983). However, the smaller substrates may not reflect the true structure of endogenous chitin. Thus, it is difficult to obtain a substrate that has the native structure of chitin and at the same time provides the susceptibility required to detect low levels of enzyme.

REFERENCES

Brine C. J. and Austin P. R. (1981a) Chitin variability with species and method of preparation. *Comp. Biochem. Physiol.* **69B**, 283–286.

Table 2. Hydrolysis of glycol chitin by chitinolytic enzymes

Substrate	Hydrolytic activity*		
	Endochitinase	Exochitinase	Lysozyme
Crab glycol chitin			
Native	11.43 ± 1.64	<0.1	1.41 ± 0.63
Reacetylated	13.71 ± 1.97	<0.1	0.60 ± 0.27
Insect glycol chitin			
Reacetylated	5.43 ± 1.34	<0.1	7.49 ± 1.45
Commercial glycol chitin			
A	<0.1	<0.1	13.67 ± 2.47
B	3.71 ± 0.71	<0.1	7.38 ± 0.97

*Unit = $\Delta A_{420}/\text{hr}/\mu\text{g} \times 10^3$.

Brine C. J. and Austin P. R. (1981b) Chitin isolates: species variation in residual amino acids. *Comp. Biochem. Physiol.* **70B,** 173–178.

Dziadik-Turner C., Koga D., Mai M. S. and Kramer K. J. (1981) Purification and characterization of two β-*N*-acetylhexosaminidases from the tobacco hornworm, *Manduca sexta* L. (Lepidoptera: Sphingidae). *Archs Biochem. Biophys.* **212,** 546–560.

Imoto T. and Yagishita K. (1971) A simple activity measurement for lysozyme. *Agric. Biol. Chem.* **35,** 1154–1156.

Imoto T., Johnson L. N., North A. C. T., Phillips D. C. and Rupley J. A. (1972) Vertebrate lysozymes. In *The Enzymes* (Edited by Boyer P. D.), Vol. 7, pp. 665–868. Academic Press, New York.

Koga D., Mai M. S., Dziadik-Turner C. and Kramer K. J. (1982) Kinetics and mechanisms of exochitinase and β-*N*-acetylhexosaminidases from the tobacco hornworm, *Manduca sexta* L. *Insect Biochem.* **12,** 493–499.

Koga D., Jilka J. and Kramer K. J. (1983) Insect endochitinases: glycoproteins from moulting fluid, integument and pupal haemolymph of *Manduca sexta* L. *Insect Biochem.* **13,** 295–305.

Kramer K. J., Dziadik-Turner C. and Koga D. (1983) Chitin metabolism in insects. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology: Integument, Respiration and Circulation,* (Edited by Gilbert L. I. and Kerkut G. A.), Vol. 3. Pergamon Press, Oxford (in press).

Senju R. and Okimasu S. (1950) Preparation of glycol chitin. *Nippon Nogei Kagaku Kaishi* **23,** 432–437.

Yamada H. and Imoto T. (1981) A convenient synthesis of glycol chitin, a substrate of lysozyme. *Carbohydrate Res.* **92,** 160–162.